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(54) Title: PARVOVIRUS CAPSIDS

(57) Abstract

The present invention relates to a method of producing non-infectious parvovirus capsids and to diagnostic assays and vaccines utilizing same. The invention also relates to a method of packaging and delivering genetic information utilizing the non-infectious capsids.

PARVOVIRUS CAPSIDS

BACKGROUND OF THE INVENTION

Technical Field

The present invention relates, in general, to a method of producing parvovirus antigens, and in particular, to a method of producing empty, and thus non-infectious, parvovirus capsids, and to diagnostic assays and vaccines utilizing same. The invention also relates to a method of packaging and delivering genetic information using the empty parvovirus capsids.

Background Information

Parvoviruses are common agents of animal disease. The first strong link between parvovirus infection and human disease came from the serendipitous discovery in 1975 of parvovirus-like particles in the sera of normal human blood donors (one of the samples having been designated B19). Since that time, B19 parvovirus has been identified as the causative agent of: i) the transient aplastic crisis (TAC) of hemolytic disease; ii) the common childhood exanthem called fifth disease; iii) a polyarthralgia syndrome in normal adults that may be chronic and resembles in its clinical features, rheumatoid arthritis; iv) some cases of anemia and/or neutropenia; and v) some cases of hydrops fetalis. The entire spectrum of human illness caused by parvoviruses, however, is not yet clear due, in large part, to the fact that an appropriate assay is not available.

Because of the very limited size of parvoviruses (about 5 kilobases), these viruses require replicating cells for propagation, and parvovirus infection, therefore, results in pathologic changes in mitotically

active host tissue. In infected children and adults, B19 parvovirus replicates in the bone marrow; in the fetus, B19 parvovirus replicates in the liver. Erythroid progenitor cells are the only cell type known to be subject to infection by this virus.

The limited host range of B19 parvovirus has hampered the development of assays specific for the virus. Since the discovery of the virus, the quantity of B19 antigen available as a reagent has been limited to that obtainable from sera of infected patients. The replication of the B19 parvovirus has recently been effected in human bone marrow cell cultures (Ozawa et al Science 233:883 (1986)). The bone marrow cultures, however, require explanted bone marrow cells and, therefore, are not practical for virus propagation. The development of and availability of clinical assays continue to be limited by the availability of the antigen. The production of stable transformants capable of producing B19 protein products has been prevented by the fact that some of these products are lethal to transfected cells.

Summary of the Invention

It is a general object of the invention to provide a method of producing large quantities of parvovirus antigens.

It is a specific object of the invention to provide a method of effecting the expression of parvovirus structural proteins in cell culture.

It is another object of the invention to provide non-infectious parvovirus capsids.

It is a further object of the invention to provide a safe and effective method of producing

antibodies against parvovirus capsid proteins.

It is a still further object of the invention to provide a vaccine effective against parvovirus infection.

It is another object of the invention to provide diagnostic assays for detecting the presence in biological samples of parvovirus particles or antibodies thereto.

It is a further object of the invention to provide a method of treating hemoglobinopathies, enzyme deficiency states and other diseases that may be amenable to genetic therapy.

Further objects will be clear to one skilled in the art from the following detailed description of the present invention.

In one embodiment, the present invention relates to a method of producing parvovirus capsids comprising the steps of:

i) introducing into a host cell a recombinant DNA molecule comprising:

a) an expression vector, and

b) a DNA sequence encoding the structural proteins of a parvovirus, with the proviso that genes encoding non-structural parvovirus proteins are not included in the DNA sequence;

ii) culturing the cells under conditions such that the structural proteins are produced and self assemble to form the capsids; and

iii) isolating the capsids.

In another embodiment, the present invention relates to a parvovirus antigen consisting essentially of a parvovirus capsid.

In yet another embodiment, the present invention relates to a diagnostic assay for parvovirus infection comprising:

i) contacting a sample from a patient suspected of being infected with parvovirus with the above-described parvovirus capsid, and

ii) detecting the formation of a complex between anti-parvovirus antibodies present in the sample and the parvovirus capsid.

In another embodiment, the present invention relates to an anti-parvovirus vaccine comprising the above-described parvovirus capsid and a pharmaceutically acceptable carrier.

In another embodiment, the invention relates to a method of packaging and transferring genetic information comprising:

i) encapsidating the genetic information in the above-described parvovirus capsid and

ii) introducing the encapsidated information into a host cell.

In yet another embodiment, the present invention relates to a diagnostic kit comprising:

i) the above-described parvovirus capsid; and

ii) ancillary reagents.

Brief Description of the Drawings

Figure 1. Human DHFR minigene DM14.

Figure 2. Structure of the B19 capsid expression vector.

Figure 3. Amplification of B19 capsid genes.

Figure 4. Immunoblot of B19 capsid proteins in CHO and bone marrow cells.

Figure 5. Immunofluorescence of a capsid--producing Chinese hamster ovary (CHO) cell-line: Figure 2A - control CHO cells, and Figure 2B transformed CHO cells.

Figure 6. Sedimentation of B19 capsids.

Figure 7. Electron micrograph of transformed CHO cells - demonstration of intranuclear viral particles.

Figure 8. Growth curves.

Detailed Description of the Invention

The present invention relates to a method of producing parvovirus structural proteins utilizing recombinant DNA techniques, to expression vectors containing DNA sequences encoding the structural proteins, and to cells transformed with such recombinant molecules. The invention also relates to diagnostic assays utilizing the recombinantly produced parvovirus protein products, or antibodies to such proteins. The invention also relates to a vaccine effective against parvoviral infection comprising the recombinantly produced viral protein product. The invention also relates to methods of treating diseases amenable to genetic therapy, i.e., hemoglobinopathies and enzyme deficiency states, utilizing the recombinantly produced parvovirus protein products, specifically parvoviral capsids, in cell transfections.

The present invention developed from Applicants' discovery that empty, and thus non-infectious, parvovirus capsids can be produced in a host cell transformed with DNA sequences encoding the large and small parvovirus capsid protein species, but not the non-structural proteins. The elimination of the noncapsid proteins

allows for the production of parvoviral particles, microscopically indistinguishable from infectious particles, which are incapable of killing the host cell.

In one embodiment, the present invention relates to a method of producing parvovirus structural proteins for example, B19 structural proteins, utilizing recombinant DNA techniques. Advantageously, the structural proteins self assemble in the host cell (eucaryotic or procaryotic) to form an empty, but intact, parvoviral capsid. Quantities of parvovirus capsids equal to or greater than those present in infected bone marrow cells, can be produced by the method of the invention.

In a preferred embodiment, eucaryctic cells are transfected with a recombinant DNA molecule comprising an expression vector and the coding sequences of the capsid proteins of a parvovirus, under control of a promoter. For selection, cells carrying a marker that alters the phenotype of the cell are used as the host. The recombinant DNA molecule containing the capsidencoding sequences is cotransfected with the sequence encoding the marker gene (i.e., a gene encoding an enzyme deficient in the untransfected cell). Transformants having the appropriate phenotype are readily selected by growing the cells in a selective medium. (Cells can be selected positively or negatively; negatively by the presence of a gene conferring resistance in selective medium and positively by the expression of a detectable marker allowing for identification and isolation of positive cells.) Such transformants are then screened, using known techniques, to determine which contain the capsid proteins. The capsid proteins are isolated in

substantially pure form using protocols known in the art.

In a most preferred embodiment, CHO cells deficient in dihydrofolate reductase (DHFR) are cotransfected with: i) a recombinant DNA molecule comprising an expression vector and a DNA sequence encoding the two B19 parvovirus capsid proteins driven by the strong single B19 promoter, and ii) a human DHFR minigene driven by the SV40 early promoter enhancer unit. Transformants bearing the DHFR+ phenotype are selected by growing the cells in a medium lacking nucleosides; colonies are screened by RNA Northern analysis for expression of B19 genes. Coamplification of the integrated B19 capsid-encoding sequence and the DHFR sequence can be accomplished by treating the cells with increasing concentrations of methotrexate; coamplification results in detectable levels of protein expression.

Empty B19 parvovirus capsids are found in the nuclei and cytosol of the CHO cells transfected and cultivated as described above. Large quantities of capsids are not released into the culture supernatants. The expression of the empty capsids does not affect growth of the CHO cells.

In another embodiment, the present invention relates to a safe and effective method of producing antibodies against parvovirus capsids. The method comprises immunizing a mammal with the non-infectious, empty parvoviral capsids described above, using protocols known in the art, and isolating the antibodies produced. Monoclonal antibodies specific for the parvoviral capsid can also be produced and isolated using known techniques. In a preferred embodiment, the antibodies, or useful

binding fragments thereof, are specific for an epitope present on the B19 capsid.

In another embodiment, the present invention relates to a vaccine effective against parvoviral infection. The vaccine includes the empty, non-infectious capsids described above (or an immunogenically effective portion thereof), purified so as to be essentially free of other proteins (that is, so as to be safe for use as a vaccine). In a preferred embodiment, the capsids are B19 capsids.

The invention also relates to diagnostic assays and kits based thereon for detecting the presence in a biological sample of either parvoviral antigens or antibodies thereto. When parvoviral antigens are sought to be detected, antibodies specific for same, produced as described above, can be used according to known protocols to effect antigen detection. When antibodies are sought to be detected, the above-described empty, non-infectious parvoviral capsids (or portions thereof recognized by the antibody), can be used as the antigen, in accordance with known techniques. It is contemplated that immunodeficient individuals incapable of producing antibodies against parvovirus can be detected by challenging such individuals with the empty, non-infectious capsid described above and determining whether antibody is produced in response to the challenge.

The diagnostic kits of the invention comprise the above-described antibodies (or binding fragments) and/or capsid antigens and reagents, such as ancillary agents, for example, buffering agents. Where necessary, the kit can further include members of a signal-producing

system, numerous examples of which are known in the art.

In another embodiment, the present invention relates to methods for packaging and delivering genetic material to the genome of a cell. The method comprises encapsidating the genetic material sought to transferred into the empty, non-infectious parvoviral capsid described above, and introducing the capsid into a host cell under conditions such that, once inside the cell, the genetic material is released from the capsid and expressed. In a preferred embodiment, adeno-associated virus DNA is used as the vector system. (See Lebkowski et al. Mol. Cell. Biol. 8:3988 (1988) and McLaughlin et al. J. Virol. 62:1963 (1988)).

Genetic material suitable for use in such a method includes genes encoding proteins useful in the treatment of genetic defects, for example, hemoglobinopathies and enzyme deficiency states. Host cells include, for example, mammalian stem cells.

The following non-limiting Examples describe the invention in more detail.

Example I

Preparation of Recombinant DNA Molecules and Transfection of CHO Cells

The DHFR minigene employed consisted of the entire coding region of the DHFR gene and included the first intron; this construct was derived by restriction enzyme digestion and ligation from the original DHFR minigene, DM-1 (Molec. Cell. Biol. 7:2830, 1987). The promoter-enhancer and polyadenylation signals were derived from the SV40 virus. For transfection, the DHFR minigene was cloned in pUC19 (see Figure 1).

To prepare the B19 capsid expression vector, the nearly full-length B19 genomic clone pYT103c was digested with the enzymes EcoR1 and Aat and subcloned into the standard vector pLTN-1. The nonstructural region was deleted by digestion with Xbal and Smal enzymes and recircularized (see Figure 2).

CHO cells were cotransfected with DNA from two plasmid constructs, one containing the DHFR minigene and the other containing the B19 capsid genes. Transformants bearing the DHFR+ phenotype were selected by growing the cells in medium lacking nuclecsides and colonies were screened by RNA Northern analysis for expression of B19 genes. Coamplification of the integrated B19 capsid--encoding sequence and the DHFR sequence was accomplished by treating the cells with increasing concentrations of methotrexate. 3-11-5 is a cell line established as described above which expresses the B19 capsid.

Example II

DNA and RNA Analysis

DNA was prepared by conventional phenol-chloroform extraction and proteinase K digestion and RNA by the conventional guanidinium sulfate method from 3-11-5 cells before and after culture in increasing concentrations of methotrexate (final concentration = 10 @M). DNA was analyzed by Southern and RNA by Northern hybridization using pYT103c, a B19 specific labeled DNA probe (Science 233:883 (1986)). The migration on agarose gel electrophoresis of the B19 DNA from 3-11-5 cells is consistent with the size of the transfected DNA insert and that of the RNA with the transcripts expected from the right side of the virus genome (J. Virol. 61:2395 (1987)) (see Figure 3).

Example III

Comparison of B19 Capsid Accumulation by Immunoblot

3-11-5 cells were compared to normal or erythroid bone marrow cells inoculated with virus and harvested at 48 hours (the peak of virus production; Blood 70:384 (1987)). Capsid protein was detected by Western blot using convalescent phase antiserum containing high titer anti-B19 capsid protein IgG (J. Virol. 61:2627 (1987)) (see Figure 4). The amount of 58 kd and 83 kd protein in 3-11-5 cells was intermediate between that harvested from cultures of normal and erythroid bone marrow. From comparison to known standard plasma preparations, it has been estimated that each 3-11-5 cell contains between 1000-20000 capsids.

Example IV

Immunofluorescence

3-11-5 and control CHO cells were fixed with acetone and stained with human convalescent phase serum containing anti-B19 capsid antibodies followed by fluorescein-conjugated anti-human IgG (J. Clin. Invest. 74:2024 (1984)). All 3-11-5 cells show a pattern of strong and specific immunofluorescence in both cytoplasm and nuclei (see Figure 5).

Example V

Sedimentation Analysis of Capsids from 3-11-5 Cells

Capsids from CHO 3-11-5 cells were compared to viral particles from human bone marrow culture (Blood 70:385 (1987)). Proteins were labeled by exposure of cultures to 35S-methionine, the cells were lysed, and the particulate fraction obtained by centrifugation over a

40% sucrose cushion (J. Virol. 61:2627 (1987)). After suspension of the particulate fraction in a small volume of buffer, radioactively labeled capsids or virions were applied to sucrose (J. Clin. Invest. 73:224 (1984)) or cesium chloride (Science 233:883 (1986)) gradients (see Figure 6). On sucrose gradient sedimentation, empty capsids were clearly distinguished from intact virions, and isopycnic sedimentation in cesium showed a density consistent with empty capsids.

Example VI

Electron Microscopy of 3-11-5 Cells

Cells were fixed and prepared for transmission EM as described (J. Clin. Invest. 74:2024 (1984)). Characteristic clusters of 20 nm particles were observed in the nuclei of 3-11-5 cells only (see Figure 7).

Example VII

Growth Curves of 3-11-5 Cells Compared to Other CHO Cells

Cells were serially harvested from microtiter wells and manually counted. Empty capsid production does not adversely affect cell proliferation of 3-11-5 (see Figure 8).

The foregoing invention has been described in some detail by way of examples for purposes of clarity and understanding. It will be obvious to those skilled in the art from a reading of the disclosure that site directed mutagenesis can be used to alter the amino acid sequence of the above-described capsids and thereby alter the tissue specificity of the virus. Furthermore, it will be clear that the DHFR-deficient CHO cells can be used to study the effect of nonstructural parvoviral proteins on cell replication. It will also be apparent

that various combinations in form and detail can be made without departing from the scope of the invention.

The entire contents of all published articles cited herein are hereby incorporated herein by reference.

CLAIMS

1. A method of producing parvovirus capsids comprising the steps of:

i) introducing into a host cell a recombinant DNA molecule comprising:

a) an expression vector, and

b) a DNA sequence encoding the structural proteins of a parvovirus, with the proviso that genes encoding nonstructural parvovirus proteins are not included in the DNA sequence;

ii) culturing the cells under conditions such that said structural proteins are produced and self assemble to form the capsids; and

iii) isolating the capsids.

2. The method according to claim 1 wherein said parvovirus is B19.

3. The method according to claim 1 wherein said host cell is a mammalian cell.

4. The method according to claim 3 wherein said mammalian cell is a dehydrofolate reductase-deficient Chinese hamster ovary cell.

5. A parvovirus antigen consisting essentially of a parvovirus capsid.

6. The parvovirus antigen according to claim 5 wherein said parvovirus is B19.

7. A diagnostic assay for parvovirus infection comprising:

i) contacting a sample from a patient suspected of being infected with parvovirus with said parvovirus antigen according to claim 5, and

ii) detecting the formation of a complex between anti-parvovirus antibodies present in said sample and said parvovirus antigen.

8. The assay according to claim 7 wherein said parvovirus is B19.

9. The assay according to claim 7 wherein said sample is a serum sample.

10. An anti-parvovirus vaccine comprising said parvovirus antigen according to claim 5 and a pharmaceutically acceptable carrier.

11. The vaccine according to claim 10 wherein said parvovirus is B19.

12. A method of packaging and transferring genetic information comprising:

i) encapsidating said genetic information in said parvovirus capsid according to claim 5; and

ii) introducing said encapsidated information into a host cell.

13. The method according to claim 12 wherein said parvovirus is B19.

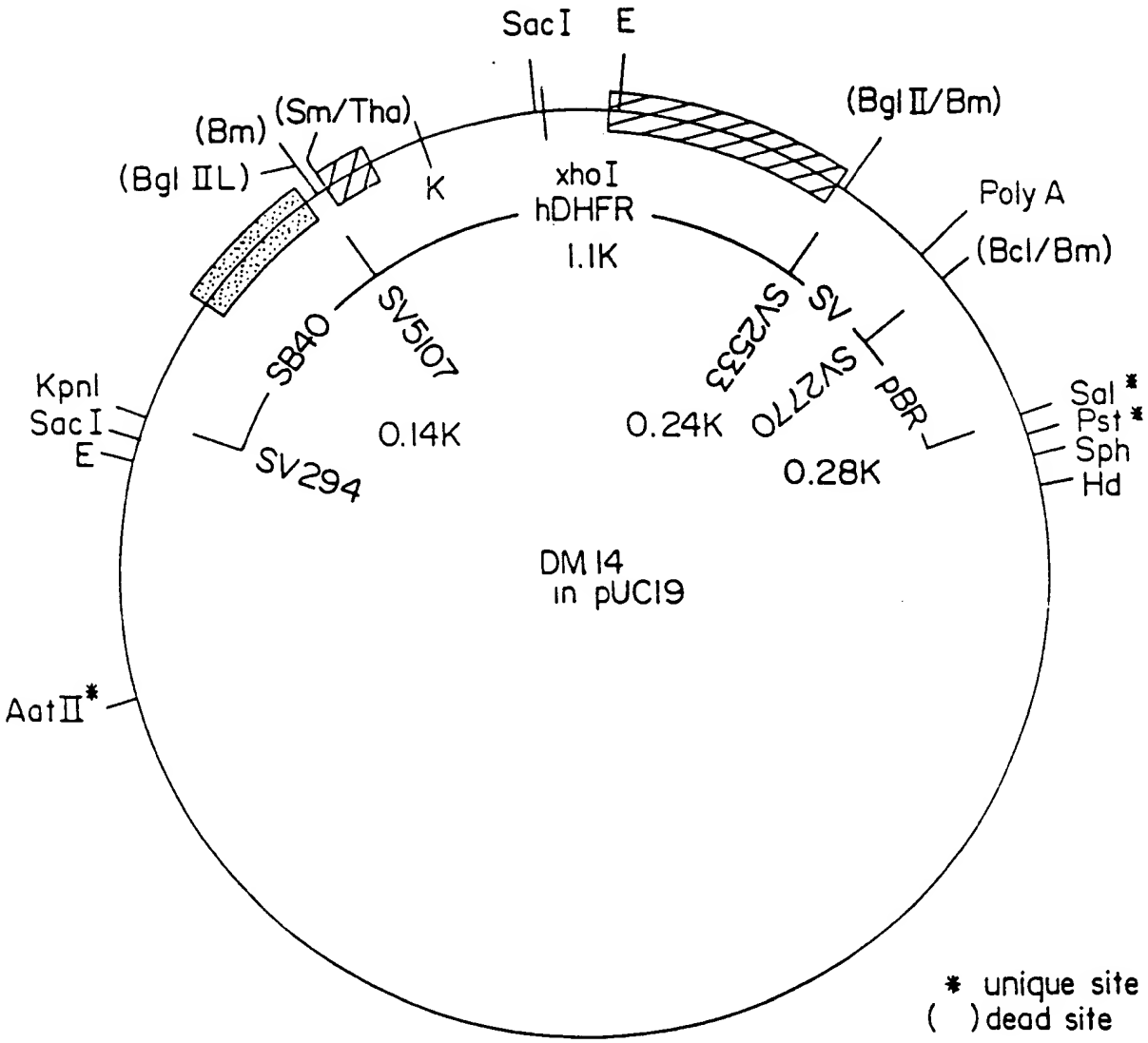
14. A diagnostic kit comprising:

i) said parvovirus antigen according to claim 5; and

ii) ancillary reagents.

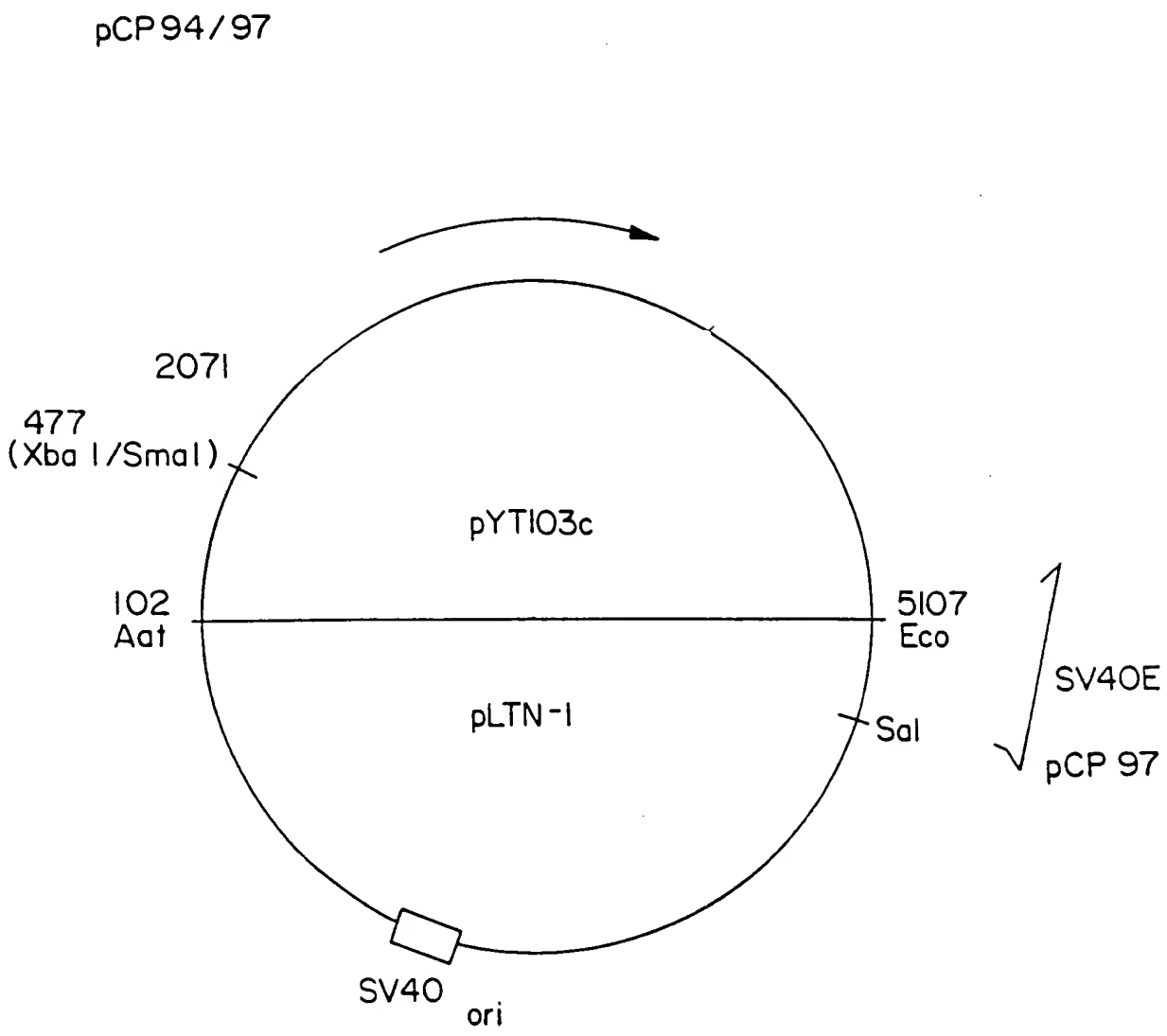
15. The diagnostic kit according to claim 14 wherein said parvovirus is B19.

16. The diagnostic kit according to claim 14 further comprising a signal-producing system.



{ SV40 early promoter/enhancer
hDHFR ex.1, Int.1, ex. 2-6
SV40 late polyadenylation

FIG. 1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477
(Xba I/SmaI)
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Aat
5107
Eco
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Sal
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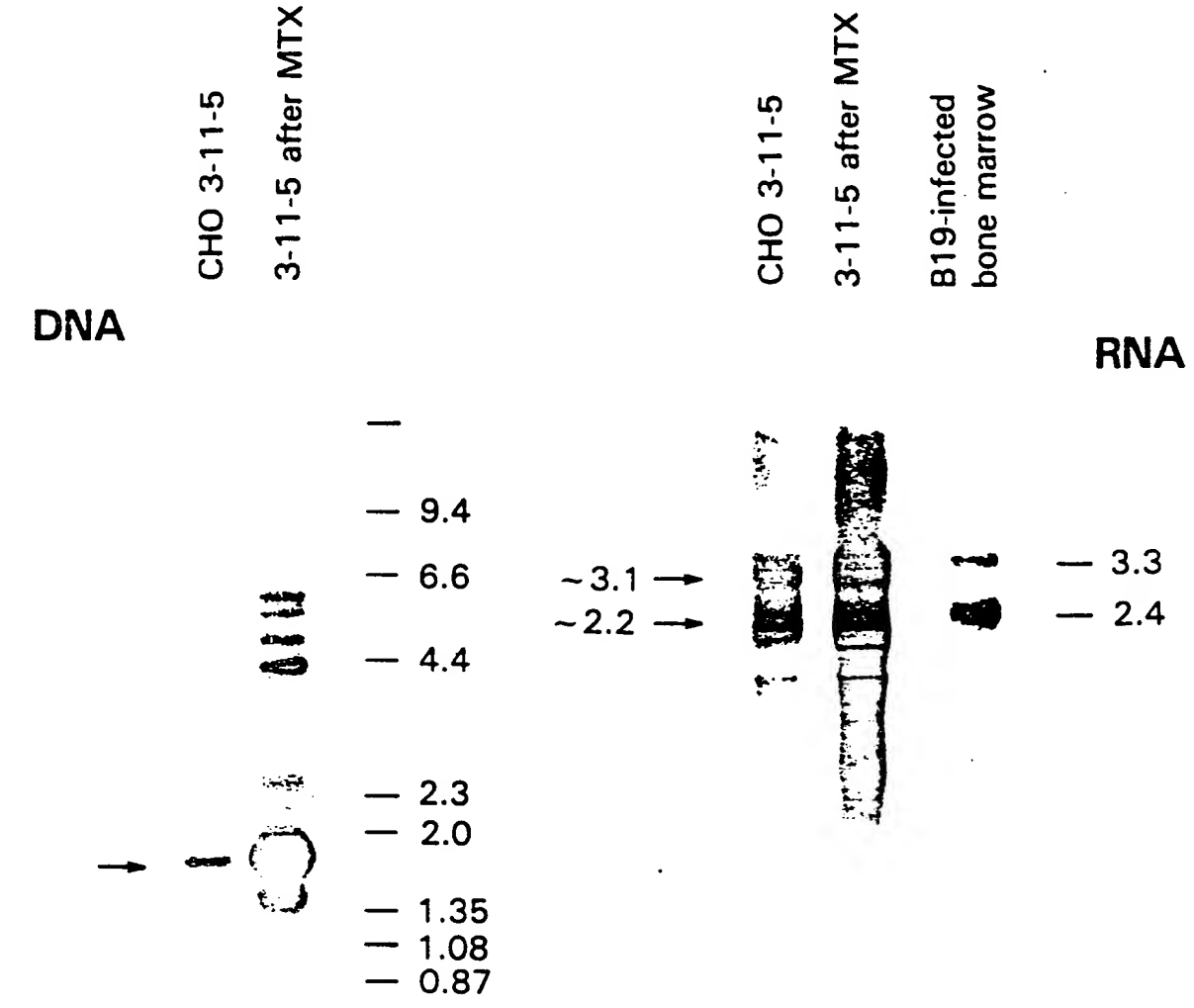
SV40
ori

FIG. 2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6.6
4.4
2.3
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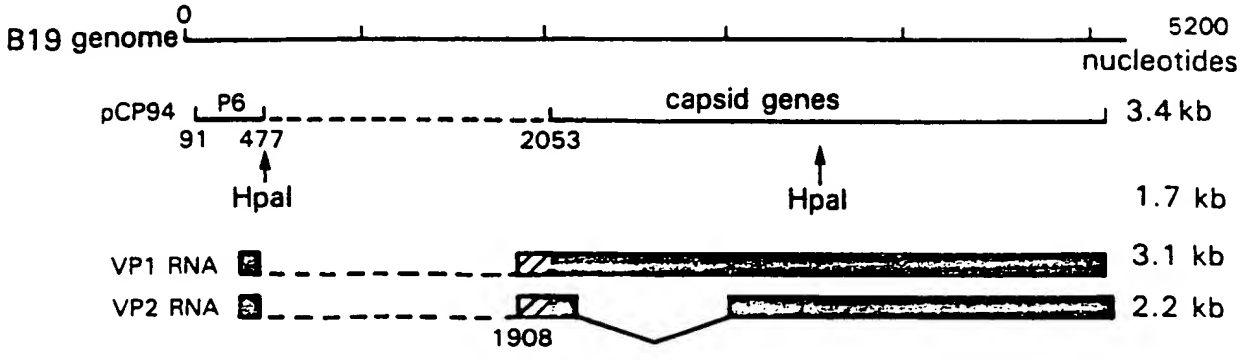
B19-infected bone marrow
RNA
~3.1
~2.2
3.3
2.4


B19 genome
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5200
nucleotides
pCP94
P6
capsid genes
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2053
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HpaI
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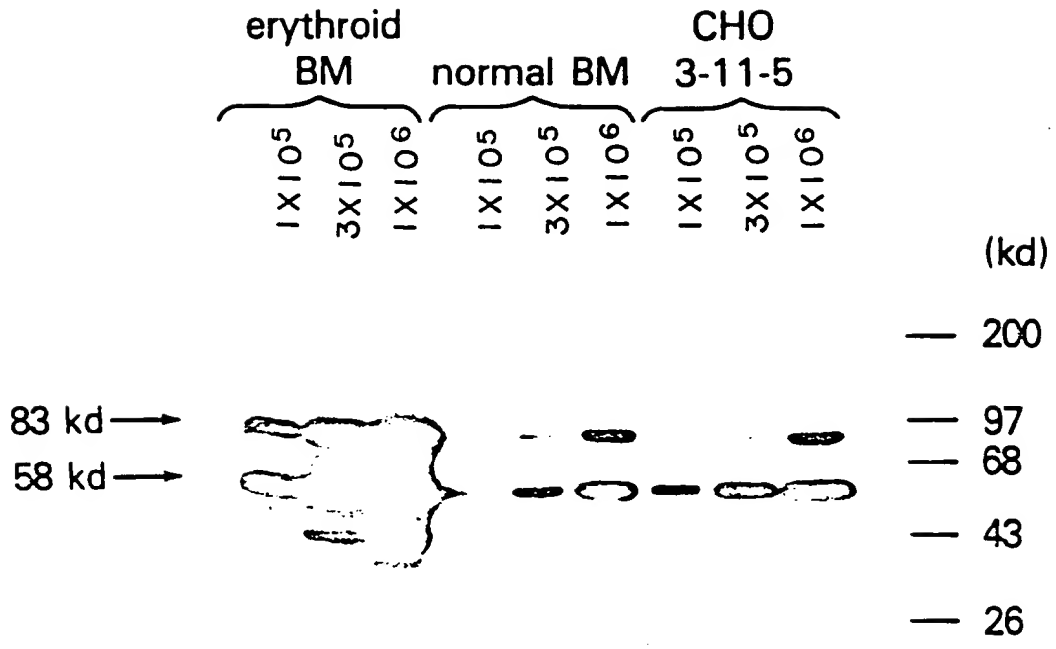
VP1 RNA
3.1 kb
VP2 RNA
2.2 kb
1908

FIG. 3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normal BM
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FIG. 4

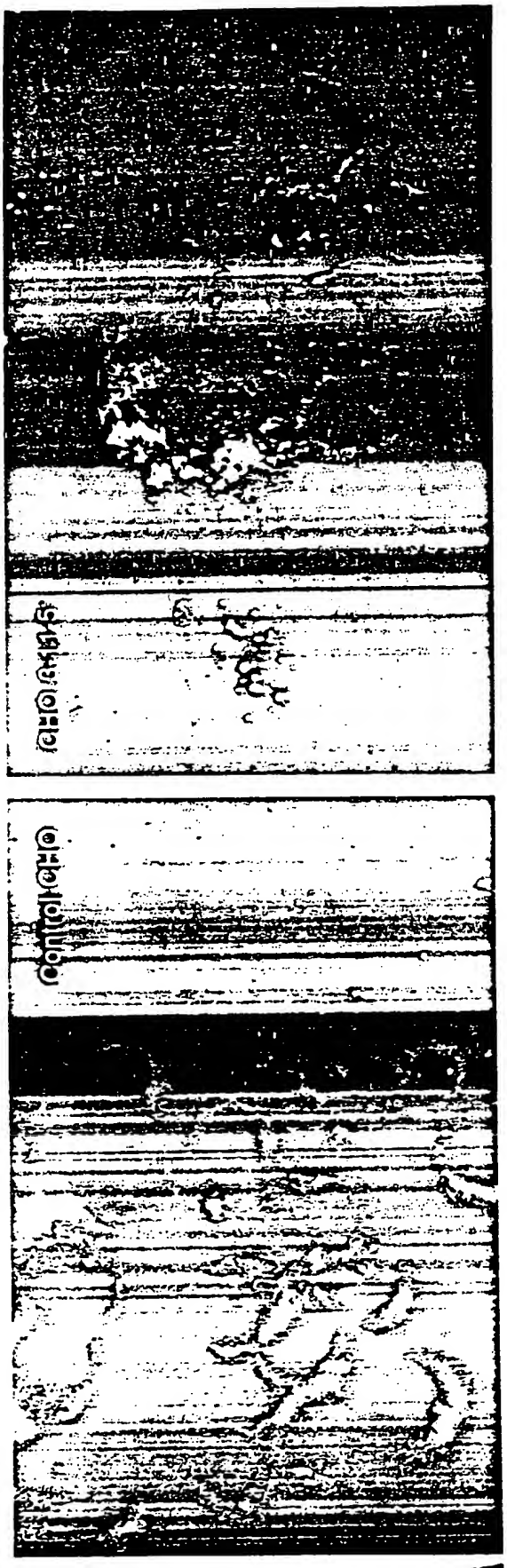


FIG. 5

FIG. 6A

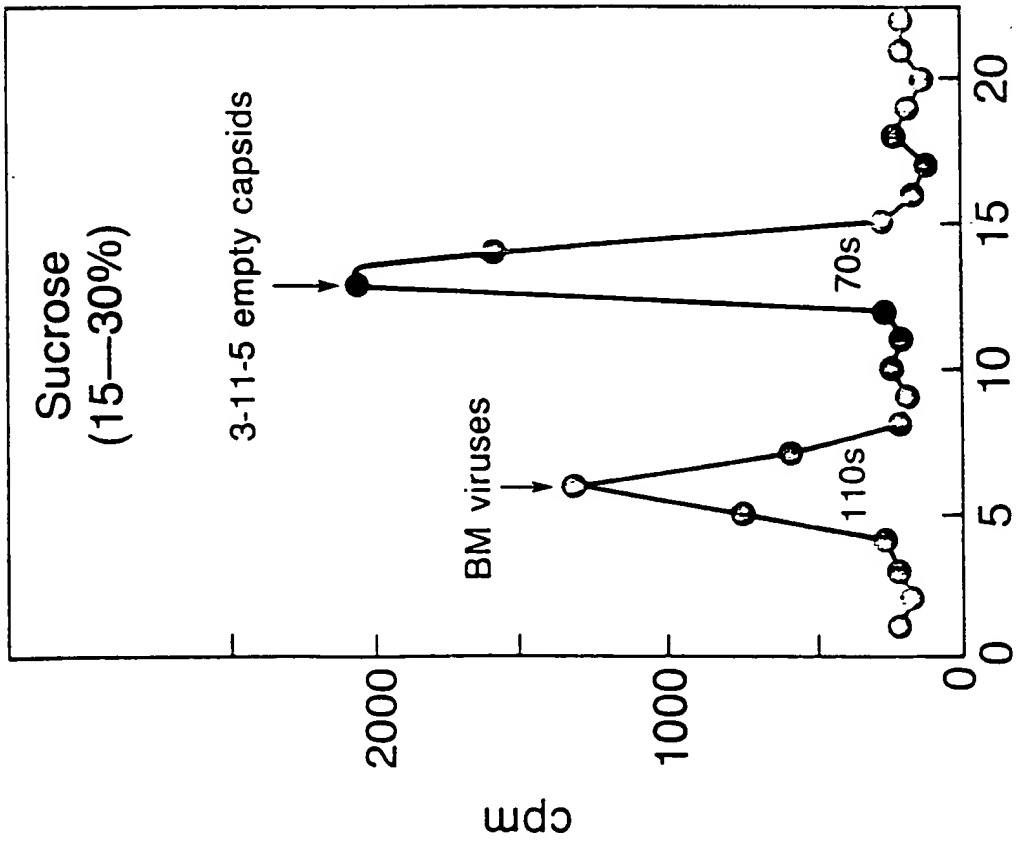
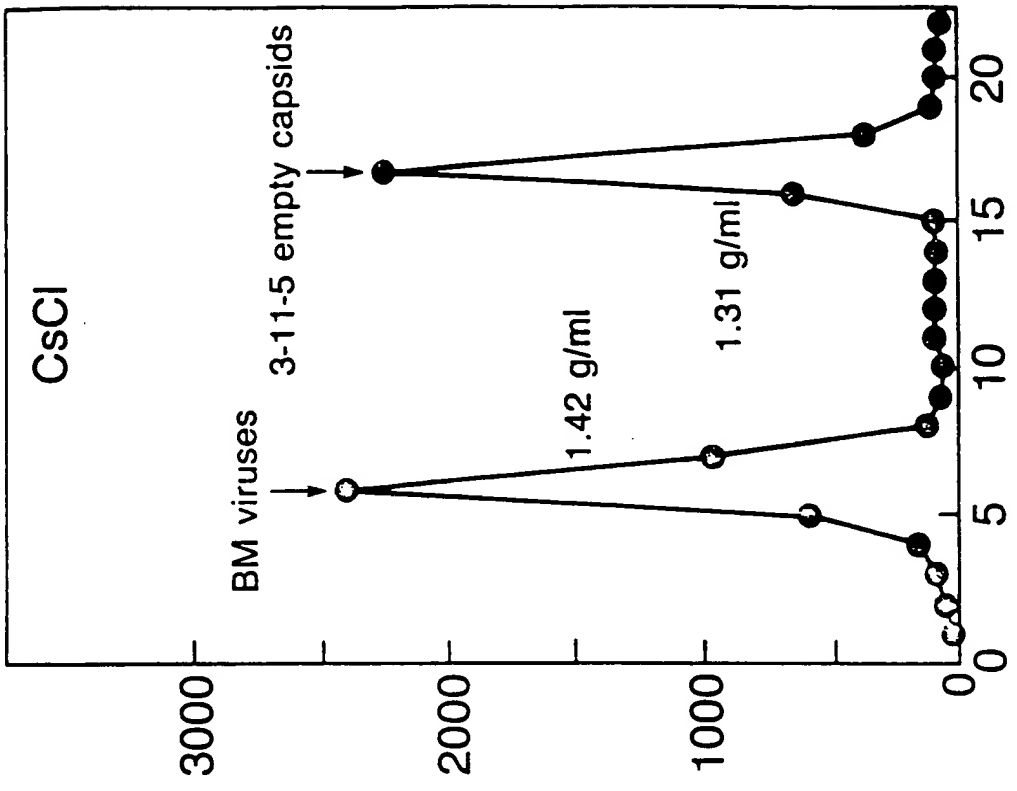


FIG. 6B



cpm

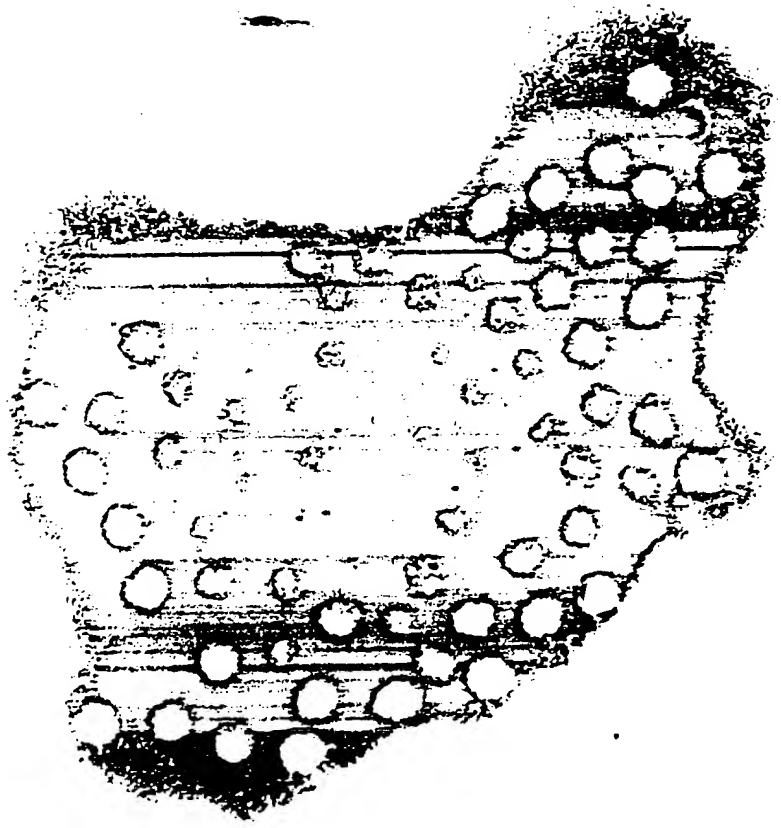


FIG. 7

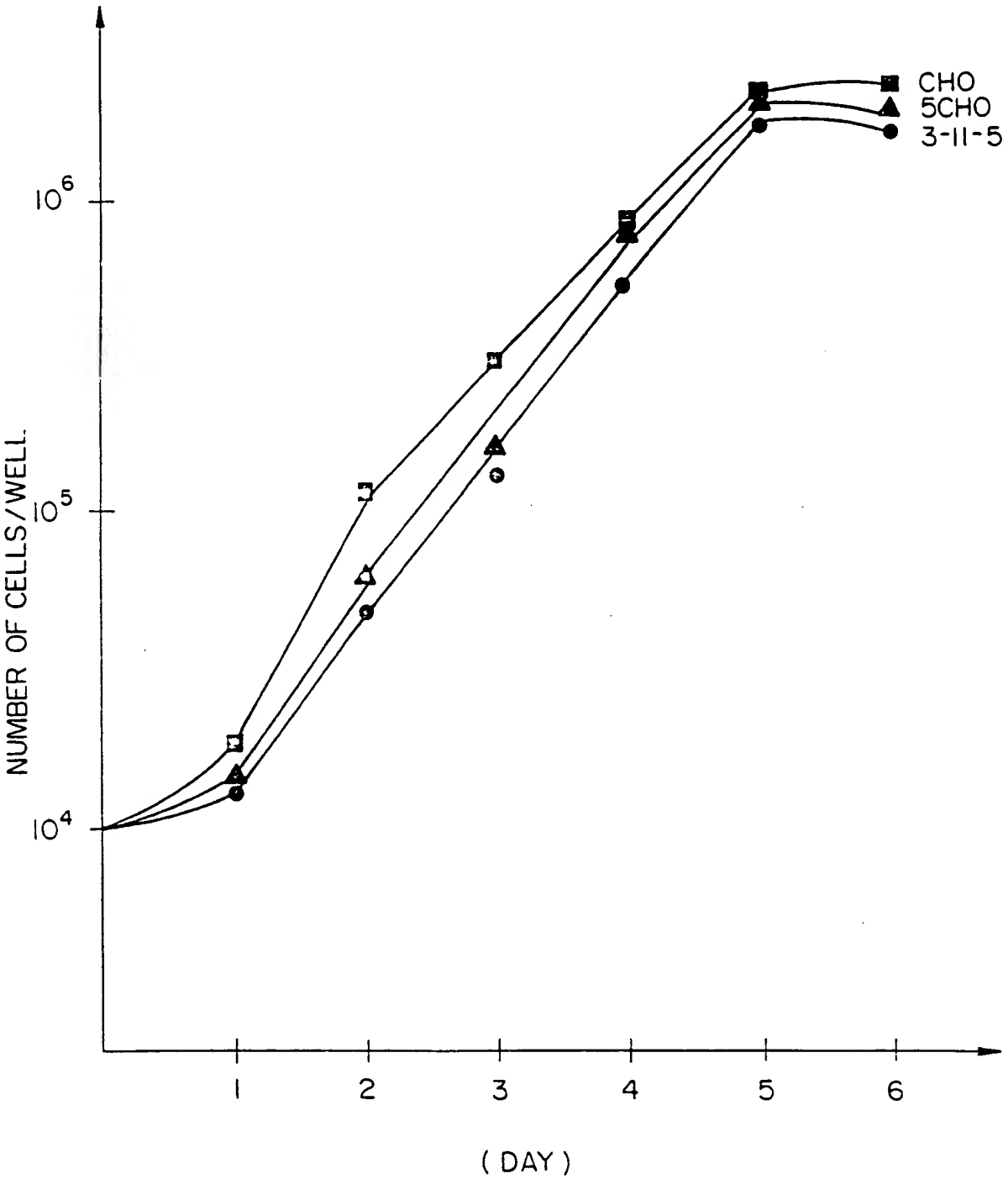


FIG. 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/04948

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/00; G01N 33/53; C12P 21/00; C12N 15/00; 7/00
C07K 13/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/69.3,70, 172.1, 172.3, 235 , 68.1 435/7; 530/350; 424/88

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chem. Abs. DataBase 1967-1990 Keywords: capsids, parvovirus, packag?, gene, empty.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Ozawa, K. et al. (August 1987) J. Virology, Volume 61:2627-2630. "Characterization of Capsid and Non Capsid Proteins of B19 Parvovirus Propagated in Human Erythroid Bone Marrow-Cell Cultures." See entire article.	1-16
Y	Cotmore, S. et al. (November 1986) J. Virology, Volume 60:548-557. "Identification of the Major Structural and Nonstructural Proteins Encoded by Human Parvovirus B19 and Mapping of Their Genes by Procaryotic Expression of Isolated Genomic Fragments". See entire article.	1-16
Y	Cochran M et al., WO 87/04463 A1, 30 July 1987, Syntro Corp. See entire application.	1-16

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 February 1990	28 FEB 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Anne Brown

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Mazzara, G et al, WO 88/02026 A1, 24 March 1988, Applied Biotechnology, Inc. See entire application.	1-10
Y	Chemical Abstracts, Volume 106(9) issued 3 March 1987 (Columbus, Ohio, USA), "Kosturko, L. et al "In vitro encapsidation of plasmid DNA into human adenovirus empty capsids". Abstract Number 61945r, Virus Res. 6(2): 123-32 (1986).	12,13
Y	Chemical Abstracts, Volume 92(23) issued 9 June 1980. (Columbus, Ohio USA) Myers, MW et al "Assembly of adeno-associated virus" Virology 102(1) 71-82 (1980). Abstract Number 194226w.	12,13